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DEVELOPMENT OF SPATIAL ORGANIZATION IN PALLEAL BUDS OF THE COMPOUND ASCIDIAN, *SYMPLEGMA REPTANS**

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Abstract

The continuous observation of living animals and the reconstruction of serial sections of fixed specimens have revealed 15 developmental stages and their timetable in palleal buds of the compound ascidian, *Symplegma reptans*. The bud primordium (stage 1) appeared on the right anterior region of the atrial epithelium of its parental bud (stage 9). It evaginated to form a vesicle along with the epidermis (stage 3) about two days later (at 18°C). Stage 4 was characterized by the formation of a test vessel through which the bud received active bloodflow. Body axes and bilateral asymmetry first became visible at this stage. Rudiments of the neural complex, pharynx, gut, and endostyle were formed directly from the inner vesicle of the bud. The neural complex placode was the first organ rudiment observed, in 3.5-day-old buds (stage 5). It changed into a tube sac whose anterior half had cilia on the luminal surface (stage 9, eight days old); the ciliated duct thus formed. In 6-day buds (stage 8), a large cell mass could be observed histologically beneath the neural complex. The cell bodies were soon arranged at the periphery of the cell mass, thus forming the dorsal ganglion. Rudiments of the pericardium, gonad, and pyloric duct appeared first as small aggregates of mesenchymal cells at stages 7, 8, and 11 (12 days old), respectively. Muscle precursors appeared in the mesenchymal space in association with the epidermis at stage 11. They differentiated into the musculus sphincter and longitudinal body musculature during stage 12, at which time, too, the stigmata perforated the pharyngeal wall. Zooids began to feed and thus attained functional maturity after about two weeks of development from buds.

Introduction

In botryllid and polystyelid ascidians palleal buds are formed by evagination of both the epidermis and peribranchial epithelium of the parental zooid (Berrill, 1940, 1941, 1947, 1948; Abbott, 1953; Fujimoto and Watanabe, 1976; Kawamura and Watanabe, 1982a). Since *situs inversus* zooids were experimentally induced in *Botryllus schlosseri* (Sabbadin, 1956), palleal buds have been used as excellent materials to investigate the development of body axes and body pattern (Izzard, 1973; Sabbadin *et al.*, 1975; Kawamura and Watanabe, 1982b, c; Nakauchi and Sugino, 1984). Recent works (Kawamura and Watanabe, 1983; Kawamura, 1984) have shown that in *Polyandrocarpa misakiensis* the anteroposterior (A-P) axis of a bud is determined with the help of positional information in the parental mantle wall. In contrast with increasing experimental works, descriptive studies on the development of zooid organization have not advanced beyond Izzard's work (1973), although fragmentary knowledge is accumulating (*e.g.*, Nunzi *et al.*, 1979; Kawamura and Nakauchi, 1984).

Symplegma reptans is one of the common botryllid ascidians in Japanese waters.

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* This paper is dedicated to the memory of the late Professor Donald P. Abbott, with deep respect.

TABLE I

Developmental stages of palleal buds in Symplegma reptans *based on living and fixed specimens*

Stage	Living materials	Fixed materials
0	Bud primordium	
1	Maximal size and evagination of bud primordium	
2	Formation of hemisphere	Proximo-distal gradient of cell height
3	Formation of closed vesicle, appearance of long axis	
4	Evagination of epidermis (tv_2), rupture of epidermal stalk (tv_1), appearance of body axes (concavities and convexities in inner vesicle)	Bilateral asymmetry of cell thickening
5	Neural complex placode forms	Presumptive domain of gut rudiment
6	Development of atrial folds and posterior projections	Establishment of budding zone
7	Formation of gut rudiment	Appearance of pericardium and gonadal cell mass
8	Pericardium, gonadal rudiment, and bud primordium appear	Dorsal ganglion cells, endostylar placode
9	Evagination of first bud, appearance of siphon anlagen	Ciliated duct, completion of pharyngeal wall, elaboration of gut rudiment
10	Appearance of endostyle	Anlagen of stigmata
11	Heartbeat begins, tv_3 and tv_4 form	Cell mass of pyloric duct
11′	Normal heartbeat	
12	Appearance of stigmata	Formation of body musculature
13	Stigmatal ciliary movement, functional maturity	
14	Sexual maturity	
15	Degeneration	

Its availability for the study of developmental biology has been pointed out repeatedly by the junior author of this paper (*cf.,* Sugimoto and Nakauchi, 1974; Nakauchi, 1976). This species is very transparent, easy to handle, and amenable to experimental manipulation (Sugimoto and Watanabe, 1980). Unfortunately, a full account of bud development in *Symplegma* has never been reported, although several workers have suggested that the basic strategy of body patterning is similar to that of *B. schlosseri* (Berrill, 1940; Sugimoto and Nakauchi, 1974; Kawamura and Nakauchi, 1976; Kawamura and Watanabe, 1982b).

The purpose of this study is to give a detailed account of the development of palleal buds in *S. reptans.* This has been accomplished by the daily observation of living animals and histological study of fixed animals. Here we present the developmental stages and timetable and report new findings in ascidian budding. We also present comparative analysis of budding in *S. reptans* as compared with blastogenesis in other botryllids and polystyelids.

MATERIALS AND METHODS

Colonies of *Symplegma reptans,* which had been cultured in the bay in the vicinity of the Usa Marine Biological Institute of Kochi University, were used as the source of larvae. Taxonomic descriptions, life history, and culture methods will be found in Tokioka (1949), Sugimoto and Nakauchi (1974), and Nakauchi (1976), respectively.

Both living and fixed specimens were studied. Observation was made on young colonies (about one week after metamorphosis) in June (seawater temperature, 20–22°C), in November (22–24°C), and in December (18–20°C). In order to observe the

TABLE II

Time schedule of bud development in Symplegma reptans *at different temperature conditions*

	Time schedule		
	Inland culture		Field
	18°C	22°C	18°–22°C
Stages	(hours)	(hours)	(days)
1	0	0	0
2	24.8	13.0	1.1
3	53.5	24.9	2.1
4	70.9	36.0	2.9
5	78.0	46.9	3.4
6	97.0	54.7	4.0
7	112.3	61.3	5.1
8	133.6	73.0	6.2
9	156.0	81.3	7.7
10	202.5	96.0	9.3
11	231.9	116.7	12.1
12	266.8	138.0	13.4
13	311.1	162.0	15.8

development of individual buds successively, colonies attached to a glass plate were cultured in a small tank placed in the laboratory. The seawater in the tank was changed twice each day. Observations were made on individual buds at intervals of eight hours. All the drawings were made with the aid of camera lucida. The rate of bud development of colonies in the laboratory was compared with that of colonies in the bay once a day. In order to facilitate histological preparations some colonies were cultured on polyethylene film. The materials were anethetized with menthol for 2 hours and fixed with Bouin's solution dissolved in seawater. They were whole-stained with borax carmine, then sectioned at 5 μm and stained with Delafield's hematoxylin and eosin.

RESULTS

In our study the life span of blastozooids was divided into 15 developmental stages (Table I), of which stage 14 and stage 15 are involved in sexual maturation and zooid degeneration, respectively. As those later developmental events are beyond the purpose of this study, only bud development up to stage 13 is dealt with here. The timetables of bud development at different temperature conditions are given in Table II. The major developmental events were observed to occur in sequence as follows.

Stage 0 and stage 1

The earliest morphological sign of bud formation was observed in the right anterior region of the atrial epithelium of a developing parental zooid when that zooid was at stage 8 (Figs. 1A, 2E:10–16). Cells of the presumptive bud domain gradually became thickened to a maximal thickness of about 10 μm (*cf.*, Fig. 3). The bud primordium in 5 μm sections consisted of about 20 cells. In this species 3–6 buds are formed in sequence from this thickened area (Sugimoto and Nakauchi, 1974), and so it is practically impossible to determine the very start of bud formation except for

the first bud of each series. In the present paper, therfore, stage 1 of bud development was allocated to a short period when the maximal thickness of the primordium was attained. The still-thickening bud primordium before this stage was defined as stage 0. The duration of stage 0 differs among buds, depending on their order of appearance in the series.

Stage 2

In this stage a bud hemisphere is formed by arching of the bud primordium (Figs. 2II, 4A). The protruding peribranchial wall is thickest at the distal area, and thinnest in the proximal part. The hemisphere consists of about 30 cells per 5 μm section. The luminal surface of the apical wall becomes indented (Fig. 4A). Nuclei are located on the outside of the thickened epithelium.

Stage 3

Stage 3 is characterized by the contraction of the basal area of the peribranchial hemisphere, a closed inner vesicle thus being formed (Figs. 1B, 2III). The outer and inner epithelia of the bud are derived from the epidermis and atrial epithelium of the parental zooid, respectively. The inner epithelium consists of about 50 cells per 5 μm section. Now the closed vesicle is ellipsoid in outline (Fig. 2III) with the long axis 1.2 times (outer layer) and 1.3 times (inner layer) the short axis. The long axis of the first bud is nearly parallel to the anteroposterior axis of the parental zooid in most cases, but often skewed in later buds. The inner layer is always thickened on the side corresponding to the right side of the future zooid (Fig. 2III). This difference in thickness of the inner wall persists through several subsequent stages (*cf.*, Fig. 1C).

The proximal end of the inner wall bears the atrial stalk on the left side of the long axis (Fig. 2III). The outer epidermal layer was not closed completely, but connected to the parent basally (Figs. 1B, 2III). This stalk acted as a test vessel, here referred to as test vessel 1 (tv_1). Unlike *Botryllus,* the tv_1 is not involved in the transfer of blood cells.

Stage 4

The second test vessel (tv_2) is formed during stage 4 from a diverticulum of the bud epidermis (Figs. 1C, 2IV), the tip of which fused with the common vascular system in the tunic. It was through this tv_2 that a developing bud was supplied with active blood circulation. Although Izzard (1973) observed a well-defined channel of bloodflow in a young bud of *Botryllus schlosseri,* no such channel was found in the present species. In contrast with the development of tv_2, tv_1 soon began to atrophy, and finally it was absorbed completely by the bud (Fig. $2IV^+$).

Body axes become gradually evident at this stage. The tv_2 developed into a ventral vessel of the future zooid, thus defining the dorsoventral (D-V) axis. As the vessel usually grew out toward the substratum, the D-V axis coincided with the parental axis. A few small morphological changes took place on the inner vesicle. Two concavities, the rudiment of pharyngeal fold, were formed sequentially on the right and left sides of the long axis (Figs. 1C, 2IV, IV^+). The vestigial atrial stalk remained as a small projection on the inner vesicle (Figs. 1C, 2A, IV^+) with an additional projection soon appearing on its right side. These projections developed into the right and left posterior ends of the peribranchial wall (PEP), respectively (see below). Thus, these projections and concavities define the anteroposterior axis of the developing bud which coincides roughly with the long axis. The posterior end of this axis was shifted

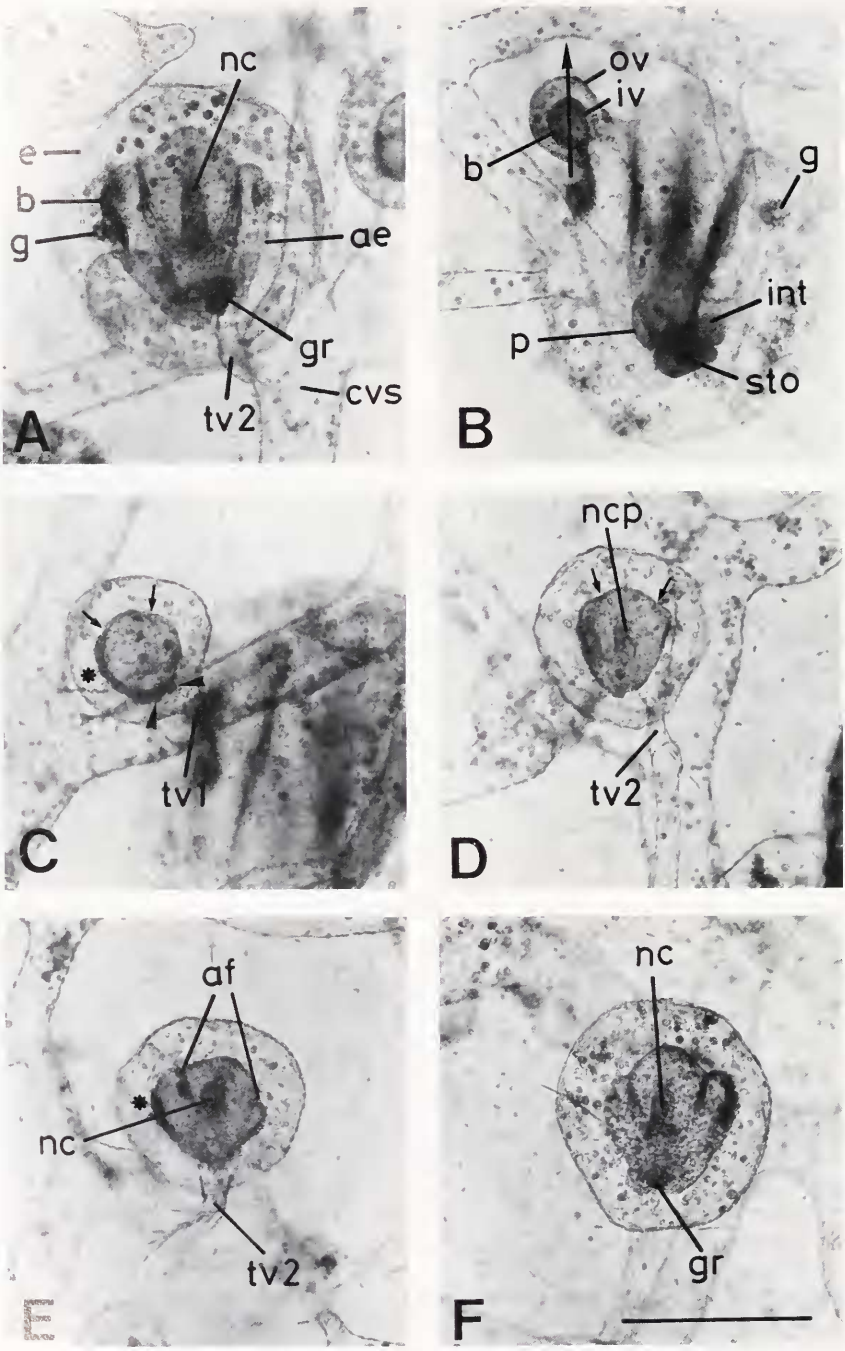

FIGURE 4. Photographs of bud development of *Symplegma reptans*, fixed whole-mounts. (A) The stage 0 bud primordium on a parental bud of stage 8, ventral view. (B) Early stage 3 bud vesicle on a parental bud of stage 10, ventral view. Arrow indicates long axis. (C) Stage 4, ventral view. Arrows in inner vesicle indicate anlagen of atrial folds. Arrowheads indicate anlagen of right and left posterior ends of peribranchial wall. Note that one side of the inner wall (asterisk) is thicker than the other side. (D) Stage 5,

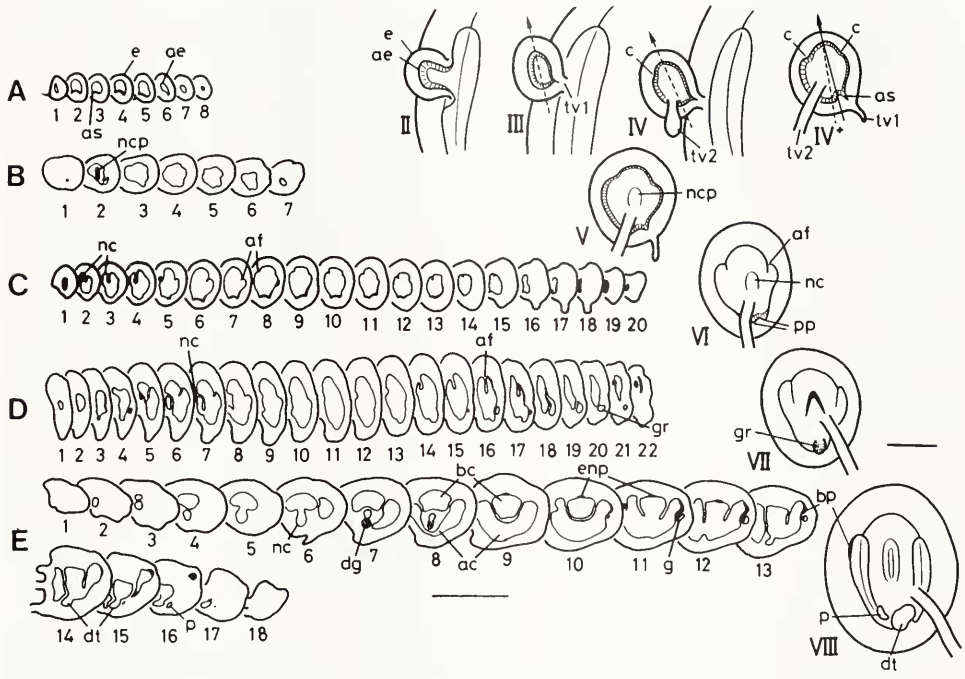

FIGURE 2. Serial sections of buds from stage 4 to stage 8 (left), and ventral views of living buds from stage 2 to stage 8 (right). Roman numerals show developmental stages. Arrow with solid shaft, anteroposterior axis; arrows with broken shaft, long axis. (A) Stage 4, from dorsal to ventral. (B) Stage 5, from dorsal to ventral. (C) Stage 6, from right dorsal to left ventral. (D) Stage 7, from right to left. (E) Stage 8, from dorsal to ventral. ac, atrial chamber; ae, atrial epithelium; af, atrial fold; as, atrial stalk; bc, branchial chamber; bp, bud primordium; c, concavity; dg, dorsal ganglion; dt, digestive tract; e, epidermis; enp, endostylar placode; g, gonad; gr, gut rudiment; nc, neural complex; ncp, neural complex placode; p, pericardium; pp, posterior projection; $tv_{1,2}$, test vessel 1, 2. Bars = 0.2 mm.

somewhat to the left of the long axis. The mid-ventral line of *Symplegma* zooids curves leftward (toward the stomach) near the posterior end, thus showing bilateral asymmetry (*e.g.*, Fig. 3A). The origin of this asymmetry could be traced back to stage 4.

During this stage, the bud expands bilaterally and the long axis becomes unclear.

Stage 5

Stage 5 begins with the appearance of the neural complex placode (Figs. 1D, 2B). This primordium, formed as a thickened disc of cells at the center of the dorsal wall

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ventral view. Placode of neural complex appears on dorsal side. Concavities (arrows) have not yet developed. Bud is connected with the common vascular system only through tv_2. (E) Stage 6, ventral view. Atrial folds begin to invaginate. Note that right anterior region of atrial epithelium (asterisk), presumptive domain of budding zone, has already thickened. (F) Stage 7, dorsal view. Gut rudiment appears. ae, atrial epithelium; af, atrial folds; b, bud; cvs, common vascular system; e, epidermis; g, gonad; gr, gut rudiment; int, intestine; iv, inner vesicle of bud; nc, neural complex; ncp, neural complex placode; ov, outer vesicle of bud; p, pericardium; sto, stomach; $tv_{1,2}$, test vessel 1, 2. Bar = 0.5 mm.

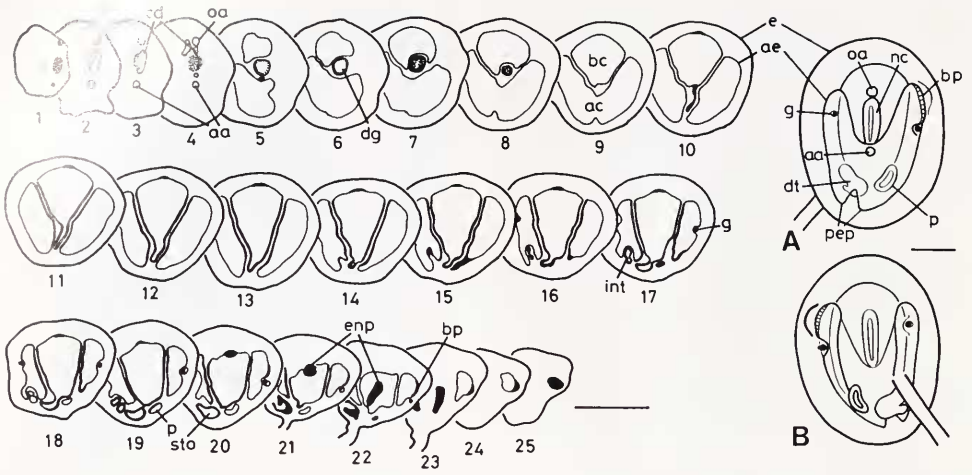

FIGURE 3. Developing buds of stage 9. (Left) Serial sections from dorsal to ventral. (Right) Living animals viewed from dorsal side (A) and ventral side (B). aa, atrial aperture; ac, atrial chamber; ae, atrial epithelium; bc, branchial chamber; bp, bud primordium; cd, ciliated duct; dg, dorsal ganglion; dt, digestive tract; e, epidermis; enp, endostylar placode; g, gonad; int, intestine; nc, neural complex; oa, oral aperture; p, pericardium; pep, posterior end of peribranchial wall; sto, stomach. Bars = 0.2 mm.

of the inner vesicle, is the first organ rudiment to appear. Cells situated between the right and left PEP anlagen became also thickened. They were the presumptive domain of the gut rudiment.

Stage 6

At the beginning of stage 6, the pharyngeal (atrial) folds originating from the concavities of the inner vesicle grow posteriorly (Fig. 1E), and the PEP anlagen become more discernible during this stage (Fig. 2C, VI). In living specimens, cells of the inner epithelial wall appeared squamous on each side of the body (Fig. 2VI). However, fixed whole mounts disclosed thickening of the right anterior region of the presumptive atrial epithelium at this and subsequent stages (Fig. 1E, F). The neural complex took on a rod-like shape (Fig. 2C:1–5). Both its ends were open to the cavity of the inner vesicle.

Stage 7

Stage 7 is recognized by the appearance of the gut rudiment (Figs. 1F, 2VII) between the two projections developing into the right and left ends of the posterior peribranchial wall. The pharyngeal folds continued to grow toward the posterior projections (Figs. 2D:5–9, 2D:14–18, 4B). A small vesicle, the eventual pericardium, appears in sections near the gut rudiment (Fig. 4B). In no case did we see the pericardial rudiment to be formed as an evagination of the inner vesicle. The dorsal ganglion was not yet established.

Stage 8

At stage 8 the pericardial rudiment can be first recognized in living specimens (Fig. 2VIII). Beginning as a single-walled vesicle, it was changed into a double-walled

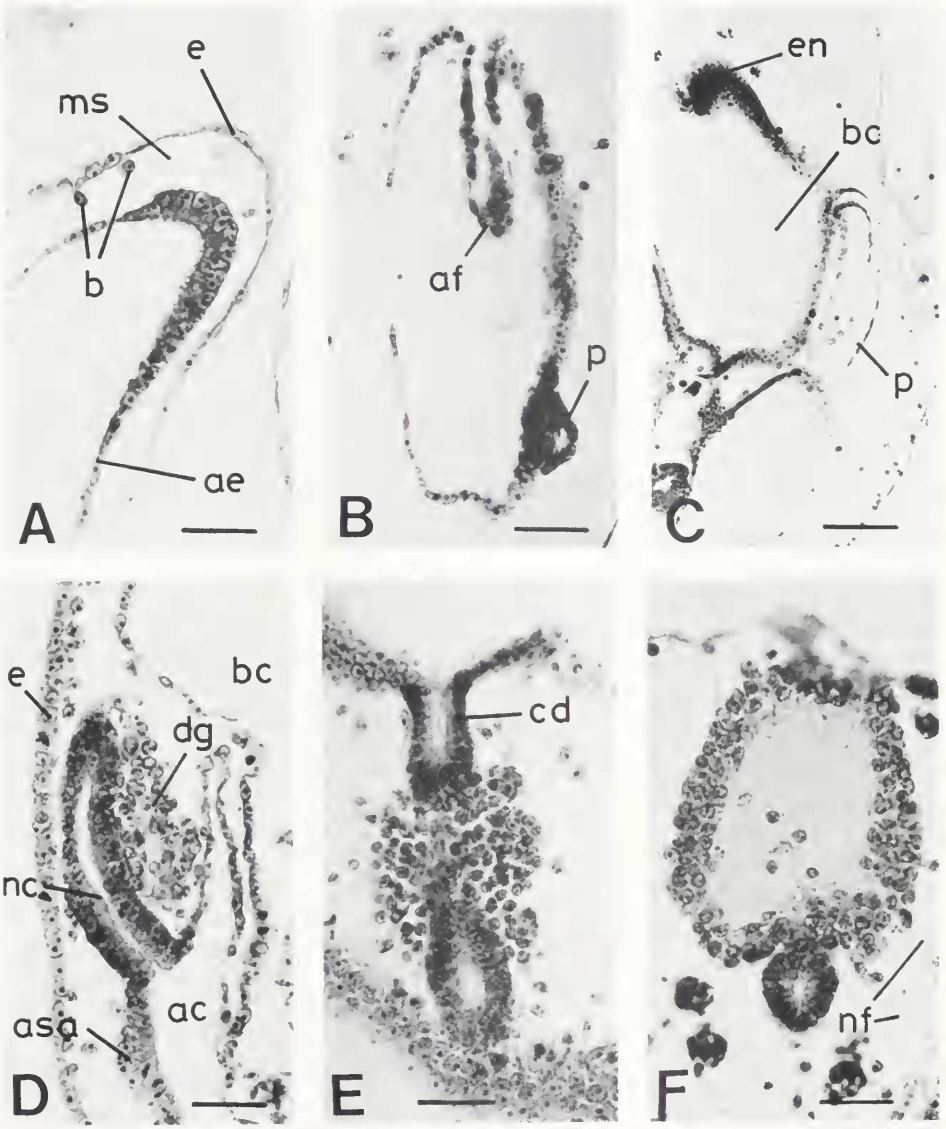

FIGURE 4. Photographs of histogenesis. (A) Protruding bud of late stage 1, frontal sectioning. Bar = 20 μm. (B) Inner wall of a developing bud of stage 8, longitudinal sectioning. Bar = 20 μm. (C) Double-walled pericardium of stage 9, frontal sectioning. Bar = 40 μm. (D) Neural complex and dorsal ganglion cells of late stage 8, longitudinal sectioning. Bar = 20 μm. (E) Neural complex of stage 9, frontal sectioning. Its anterior half is opened to the pharyngeal chamber and has cilia on the luminal surface. Bar = 20 μm. (F) Dorsal ganglion of stage 11, frontal sectioning. Bar = 20 μm. ac, atrial chamber; ae, atrial epithelium; af, atrial fold; asa, atrial siphon anlage; b, blood cell; bc, branchial chamber; cd, ciliated duct; dg, dorsal ganglion; e, epidermis; en, endostylar placode; ms, mesenchymal space; nc, neural complex; nf, nerve fiber; p, pericardium.

vesicle during this stage (*cf.*, Fig. 4C) by inward folding of the cardiac muscle cell domain. The pericardial rudiment was situated to the right of the gut rudiment, making clearer the bilateral asymmetry of the earlier stages.

The atrial (pharyngeal) folds extend to the posterior end of the inner vesicle on the ventral surface (Fig. 2E:13–15), while meeting just behind the neural complex on the dorsal surface (Fig. 2E:8), thus separating the branchial chamber from the atrial chamber. The anterior and posterior ends of the neural complex were opened into the branchial and atrial chamber, respectively (Figs. 2E:5–6, 4D). Free cells began to accumulate beneath the neural complex to form the dorsal ganglion cells. In sections the endostyle rudiment appears as a cell thickening along the mid-ventral line of the branchial floor (Fig. 2E:9–12).

By mid stage 8 of living animals a stage 0 bud primordium becomes visible on the right anterior region of their atrial epithelium (Fig. 2VIII). However, as we have already described, thickening of the right atrial epithelium could be traced back at least to stage 6 in sections or fixed whole mounts.

The gonadal rudiment, derived from a small mass of cells observed in sections of the preceding stage, became visible in living animals on each side of the body. It was located within a concavity, termed the genital cavity, formed by an invagination of the peribranchial wall (Figs. 2E:10–13, 5A, B). The dextral genital cavity was situated just posterior to the bud primordium (Fig. 5B). The sinistral one was situated a little more anteriorly than the dextral. The gonadal rudiment itself took a hollow, spherical form. A multi-layered mass of cells faced the wall of the genital cavity. They contained oocytes with the largest nuclei about 5 μm in diameter (Fig. 5A). The testis also develops from this cell mass on the dorsal side of the oocytes (not shown). On the other hand, gonadal cells facing the mesenchymal space were mono-layered (Fig. 5B). They form the brood pouch and oviduct at later stages. The gonad did not undergo substantial further morphological changes until the colony entered the sexual reproductive phase. Further development of the gonad will be described in another report.

Stage 9

At stage 9 the bud primordium begins to evaginate. Two siphon anlagen which were first seen in sections of stage 8 specimens could now be observed on the mid-dorsal line of the "parental" bud near each end of the neural complex (Fig. 3A). The branchial siphon was formed by the contact of wall of the branchial (pharyngeal) chamber with the epidermis, while the atrial siphon by the contact of wall of the atrial chamber with the epidermis (*cf.*, Fig. 4D).

The branchial chamber was now completely separated from the atrial chamber (Fig. 3:9–22). Around this stage, the neural complex lost its connection with the atrial chamber. The anterior half of the neural complex had cilia on its luminal surface, which was differentiating into the ciliated duct (Fig. 4E). Cytoplasm facing the lumen of the ciliated duct was weakly stained with eosin. In the dorsal ganglion, cell bodies became peripherally arranged, and the central matrix of the ganglion was eosinophilic (Fig. 3:5–6). The gut rudiment elongated and began segmentation into the esophagus, stomach, pyloric caecum, and intestine.

Stage 10

At the beginning of stage 10 the endostylar rudiment becomes visible in living specimens. This had already been recognized histologically at stage 8 but was not evident in living specimens until the longitudinal groove of the endostyle emerged mid-ventrally on the pharynx (*e.g.*, Fig. 6:45–48). Usually the dextral margin of the groove appeared earlier than the sinistral one (Fig. 6A, B).

The pharynx consists of two epithelial walls; one facing the branchial chamber and the other the atrial chamber. In sections, cells of the walls began to be swollen at intervals along the anteroposterior axis (*e.g.*, Fig. 6:29–32). These were the first sign of the stigmata anlagen. In the alimentary tract the stomach was especially enlarged, but not yet plicated.

The first bud of the "parental" bud had by now developed into stage 2.

Stage 11

Stage 11 begins with the start of the heartbeat. At first the heartbeat was faint and irregular. The stage where the heart beats regularly is defined as stage 11′. A parental zooid of this stage bore its own first bud of stage 4 and the second one of stage 1 (Fig. 7A).

Until stage 11 a developing zooid had only one test vessel, tv_2, but now tv_3 appeared as an evagination of the ventral epidermal wall near the posterior end and tv_4 appeared beneath the budding zone (Fig. 7). Both vessels elongated and connected with the common tunic vascular system.

The ventral edge of the branchial sac looked wavy (Fig. 7A). In sections, the outer and inner pharyngeal walls were not only thickened at intervals but also associated tightly with each other (*e.g.*, Fig. 7:21–24). These stigmata anlagen could not be detected in living specimens until stage 12. In the digestive tract, cell aggregates appeared in the vicinity of the descending limb of the intestine (Fig. 5C). During this and subsequent stages, they developed into the pyloric gland and duct (Fig. 5D). The wall of the stomach now began to show plications.

The dorsal ganglion extended nerve fibers (Fig. 4F). The luminal surface of the ciliated duct became highly eosinophilic. Single basophilic mesenchyme cells became associated with the dorsal epidermis and both siphons (Fig. 5E). They were small and spherical around the siphons, but often fusiform beneath the epidermis. These cells were the precursor of body musculature.

Stage 12

Stage 12 is defined by perforation of the stigmata. The stigmata grew from the wave-like structures of the branchial wall (Fig. 7B), each "wave" coinciding with a stigmatal row where the pharyngeal wall had been thickened at stages 10 and 11. The antero-ventral four to six rows of stigmata appeared first, and new ones were added postero-dorsally up to 7–8. The internal longitudinal vessels could first be seen at this stage. At first, only one stigma was perforated between respective vessels (Fig. 7B), and then two or three stigmata were added to form a total of 16–19 stigmata per row. It was unclear whether the perforation of stigmata was accompanied by cell dissociation or cell death of the thickened cells of the pharynx.

Muscular sphincters appeared around the oral and atrial siphons, and the longitudinal body muscles were developed just beneath the dorsal epidermis (Fig. 5F). Muscular cytoplasm was highly eosinophilic. Glandular cells containing eosinophilic granules appeared in the posterior part of the neural complex, forming the neural gland.

Stage 13

At stage 13 the cilia of the stigmata began to beat, and feeding commenced one or two days later. Thus the young zooids were functional.

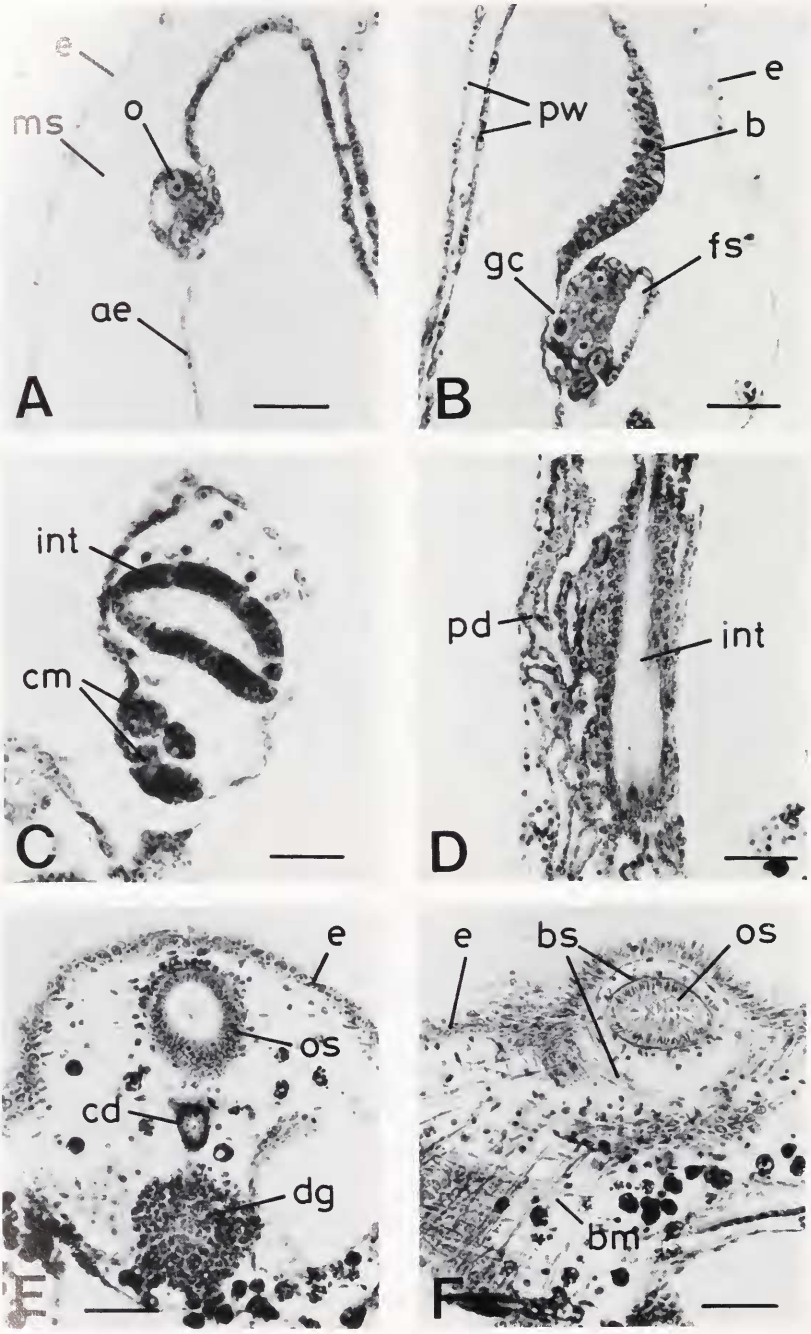

FIGURE 5. Photographs of histogenesis. (A) Sinistral gonadal rudiment of stage 8, frontal sectioning. Bar = 20 μm. (B) Dextral gonadal rudiment of early stage 9, frontal sectioning. Bar = 20 μm. (C) Descending limb of intestine, stage 11, transverse sectioning. A large number of small cell mass appear. Bar = 20 μm. (D) Intestine and pyloric duct of stage 12, frontal sectioning. Bar = 40 μm. (E) Dorsal surface of a developing zooid of stage 11, frontal sectioning. Single mesenchymal cells are associated with the epidermis

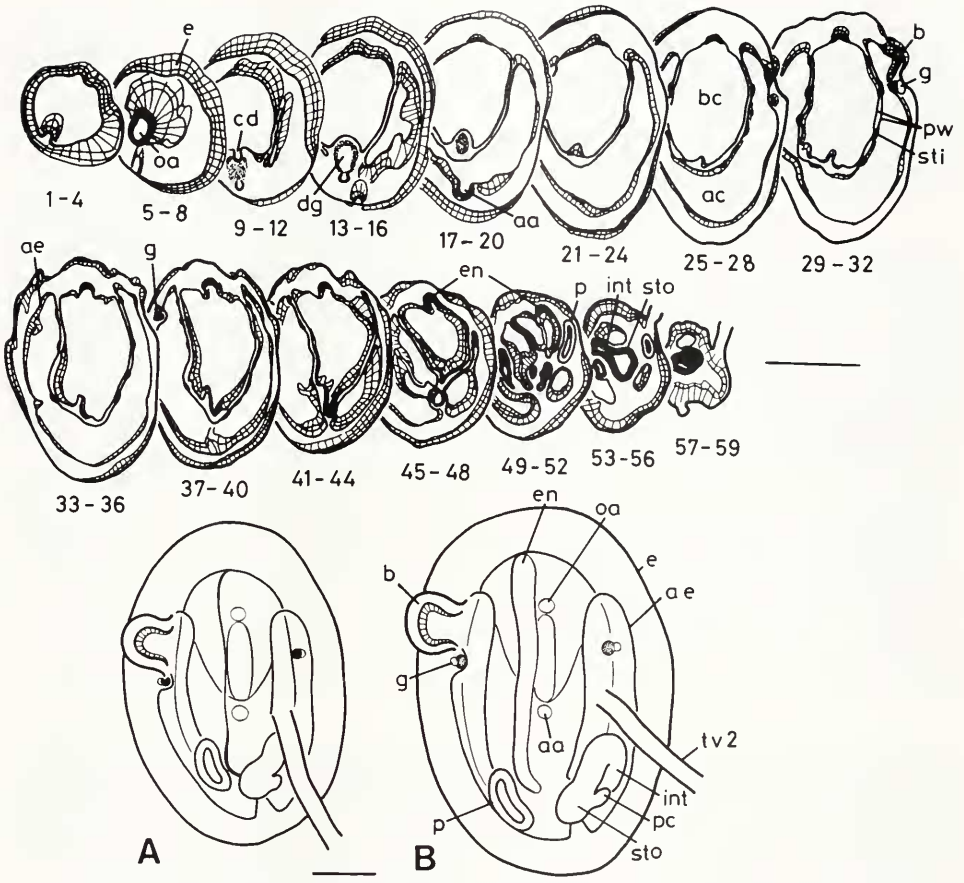

FIGURE 6. Developing zooids of stage 10. (Upper) Serial sections from dorsal to ventral. (Lower) Living animals of early stage 10 (A) and late stage 10 (B), ventral view. aa, atrial aperture; ac, atrial chamber; ae, atrial epithelium; b, bud; bc, branchial chamber; cd, ciliated duct; dg, dorsal ganglion; e, epidermis; en, endostyle; g, gonad; int, intestine; oa, oral aperture; p, pericardium; pc, pyloric caecum; pw, pharyngeal wall; sti, stigmata anlagen; sto, stomach; tv_2, test vessel 2. Bars = 0.2 mm.

DISCUSSION

In the present study, we have revealed the developmental stages of a bud and their time schedule in *Symplegma reptans*. The results give new information about the difference between *Symplegma* and other botryllid and polystyelid budding.

Formation of the bud primordium

In *Botryllus schlosseri* the bud primordium first appears as a thickened disc of cells on each side of the parental body of stage 8 (staging by Izzard, 1973). In contrast,

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and the oral siphon. Bar = 40 μm. (F) Dorsal surface of a developing zooid of late stage 12, frontal sectioning. The branchial sphincter and body muscle are developed. Bar = 40 μm. ae, atrial epithelium; b, bud primordium; bm, body muscle; bs, branchial sphincter; cd, ciliated duct; cm, cell mass; dg, dorsal ganglion; e, epidermis; fs, follicle stalk; gc, genital cavity; int, intestine; ms, mesenchymal space; o, oocyte; os, oral siphon; pd, pyloric duct; pw, pharyngeal wall.

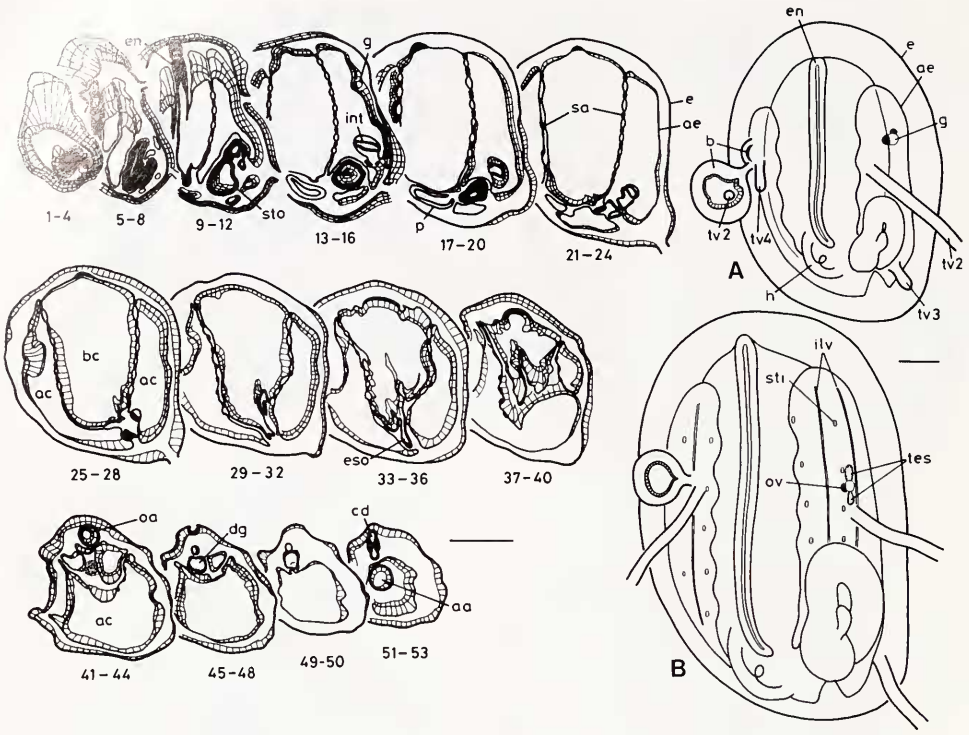

FIGURE 7. Developing zooids of stages 11 and 12. (Left) Serial sections of stage 11 zooid, from ventral to dorsal. (Right) Living animals of stage 11 (A) and stage 12 (B), ventral view. aa, atrial aperture; ac, atrial chamber; ae, atrial epithelium; b, bud; bc, branchial chamber; cd, ciliated duct; dg, dorsal ganglion; e, epidermis; en, endostyle; eso, esophagus; g, gonad; h, heart; ilv, inner longitudinal vessel; int, intestine; oa, oral aperture; ov, ovary; p, pericardium; sa, stigmata anlagen; sti, stigmata; sto, stomach; tes, testis; $tv_{2,3,4}$, test vessel 2, 3, 4. Bars = 0.2 mm.

S. reptans shows cell thickening, the first manifestation of budding, at a much earlier stage. Developing buds of stage 6 had already showed significant cell thickening on the right anterior region of the atrial epithelium. The area of thickening eventually could be traced back to the distal thickened area of a protruding bud (stage 2). Our unpublished data suggest that in *S. reptans* columnar or cuboidal cells are physiologically more active than squamous cells. These results suggest in turn that the budding activity of a developing zooid may not be acquired *de novo* but result from the transmission and maintenance of high physiological activity in the budding zone of the preceding generation. *S. japonica* in which a bud arises on each side of the parental body (Watanabe, pers. comm.) may contribute to our further understanding of the manner by which the budding domain is established.

Unlike *Botryllus* and *Botrylloides* zooids, each *Symplegma* zooid makes buds several times during its life span (Sugimoto and Nakauchi, 1974). The area of the thickened disc of *Symplegma* is not wholly involved in a single bud, but some area remains as sources of the succeeding buds (Berrill, 1940). The duration of the disc stage is, therefore, short in earlier buds but has become longer and longer in later buds. This makes it practically impossible to apply the staging of *Botryllus* (Sabbadin, 1955; Izzard, 1973) to *Symplegma* without major modification. In order to compare the

developmental timetables of successive buds, we distinguished a growing bud disc from the fully developed disc, by defining the former as stage 0. Our stage 0 and stage 1, therefore, correspond to stage 1 and stage 2 of Berrill (1941). Stage 1 as defined by Izzard (1973) covers our stages 0 and 1. Stage 0 is histologically detectable when the parental bud enters stage 6.

Development of bud polarities and bilateral asymmetry

Symplegma differs from *Botryllus* in the way the closed vesicle is formed from a hemisphere. In *B. schlosseri,* the beginning of stage 3 is recognized by the skewing of the atrial hemisphere in the sagittal plane toward the anterior end of the parental bud (Izzard, 1973). The long axis of the resultant bud vesicle is in harmony with the A-P axis of the parent, and the dorso-ventral (D-V) axis lies perpendicular to the parent in a horizontal plane (Sabbadin *et al.,* 1975). Vesicle formation in *S. reptans* varies from individual to individual. The long axis of a stage 3 bud is not always parallel with the parental A-P axis. The D-V axis that orients differently according to case, works in harmony with that of the parent by the time the bud's neural complex is formed (Kawamura and Watanabe, 1982b). However, we do not think that this difference comes from an intrinsic difference in bud formation but from the fact that *Botryllus* forms a common cloacal system and each bud is settled in a fixed position in the system at the early stage of its development. *Symplegma,* on the other hand, makes no systems and buds are able to change their position and orientation in the colony as late as stage 12. Because of this capacity for movement, a *Symplegma* bud can easily change the orientation of its long axis and D-V axis relative to those of the parent.

Previous work (Kawamura and Watanabe, 1982b) has shown that in *S. reptans* bud polarity develops as if the bud "knew" the position on the parental lateral wall from which it has arisen, a phenomenon referred to as the parental lateral effect. The present results confirm and extend our earlier findings histologically. The thickened columnar cells of the apical region of a stage 2 bud could be followed until stage 4, when the antero-posterior polarity became visible. They constituted mainly the dextral lateral wall of a developing bud. The atrial stalk that had connected the inner vesicle with the parental peribranchial wall formed a small projection and finally developed into the posterior end of the left peribranchial wall (left PEP) in the future zooid. In *B. schlosseri,* two posterior corners of inner vesicle appear in advance of the atrial folds (the pharyngeal rudiment), and the right posterior corner projects further posteriorly than the left one (Izzard, 1973). This is the first morphological expression of bilateral asymmetry. Our PEP anlagen are different from the posterior corners of *Botryllus* in their sites of appearance and in their mode of development. In *Botryllus* the posterior corners form on each side of the atrial stalk, and later the right one grows further than the left one. This unequal growth results in a skewing of the body axis toward the left side posteriorly in the future zooid. In *Symplegma,* as the left PEP anlage corresponds to the atrial stalk, the right side of the body is larger than the left side from the very start. Thus, we conclude that the skewing of body axis in *Symplegma* is associated with the asymmetrical emergence of PEPs.

In *B. schlosseri,* experimental work has suggested that bud polarity is determined by the common vascular system (Sabbadin *et al.,* 1975). In normally developing buds, the epidermal stalk serves as a blood vessel, and a channel of bloodflow is established within a bud of late stage 3 (Burighel and Brunetti, 1971; Izzard, 1973). The point where the stalk enters the bud corresponds to the posterior end of the future zooid. In *S. reptans* no active circulation could be observed between buds and the parent

through the stalk, tv_1, which atrophied at stage 4 (*cf.*, Mukai *et al.*, 1978). Instead, tv_2, which emerged ventrally at stage 4, was the first and only vessel that connected the bud with the common vascular system, until tv_3 and tv_4 appeared at stage 11. The entrance point of blood through tv_2 was the left ventral region of the future zooid. Thus, the contribution of bloodflow to body axis determination seems to be negligible in *S. reptans*.

In *S. reptans*, the posterior end of a developing bud is established in the vicinity of the atrial stalk where cells originally occupying the periphery of the bud primordium come in contact with one another at the time the protruding bud is pinched off. This means that normally nonadjacent cells are juxtaposed at the proximal end of a bud vesicle. In palleal buds of *Polyandrocarpa misakiensis*, such a juxtaposition of cells has been shown to play an important role in the establishment of anteroposterior body pattern (Kawamura and Watanabe, 1983; Kawamura, 1984). When bud pieces of *Polyandrocarpa* originating from different positions of the parental mantle wall are surgically apposed, mitotic activity is enhanced at the boundary region, resulting in the formation of the gut rudiment that is the posterior-most organ (Kawamura and Nakauchi, 1986). We are currently trying to determine whether the posterior determination in *Symplegma* buds is of *Polyandrocarpa* type.

Basic strategy of organogenesis

The pharyngeal rudiment, gut rudiment, endostyle, and neural complex are the major organ rudiments formed directly from the inner vesicle of a bud in *S. reptans*. According to Berrill (1941) and Izzard (1973), the gut rudiment is the first organ to be established in a developing *Botryllus* bud, and then the rudiments of the neural complex and pericardium follow. In *Metandrocarpa taylori*, the endostyle is one of the first structures to appear in the newly detached bud (Abbott, 1953). In *S. reptans*, the neural complex placode manifested itself at stage 5, and then the gut rudiment appeared (stage 7), well before the endostyle (stage 10). The atrial folds (pharyngeal rudiment) had not yet developed at stage 5. This precocious appearance of the neural rudiment in *Symplegma* is instructive when considered from the comparative embryological viewpoint. In colonial ascidians, zooid organization can be constructed via two ways; one from an embryo or tadpole larva and the other from an asexual bud. Presently we do not know the extent to which these two pathways share the basic strategy of organogenesis with each other. But, in this study we can find an analogy between the neural complex tube in *Symplegma* buds and the "neural tube" in ascidian embryos. In chordate embryogenesis, the neurula stage follows the gastrula. As the double-walled vesicle stage (stage 3) of ascidian palleal buds is comparable to the gastrula stage of embryos (*cf.*, Berrill, 1955), the stage 5 of *Symplegma* may be likened to a "neurula" stage. Recentry, we found that in *Botrylloides simodensis* blastogenesis (in prep.) the neural placode was the earliest organ rudiment to be established, paralleling our finding in *S. reptans*.

In *Botryllus* the neural complex arises as a tubular dorsal outpocketing of the inner vesicle (Hjort, 1896; Ritter, 1896; cited by Abbott, 1953). This outpocketing grows forward and establishes a secondary connection anteriorly with the pharyngeal cavity, the original posterior aperture soon closing permanently. In *M. taylori*, the tube arises as a folded placode which cuts off starting from the anterior end, and the definitive anterior connection with the pharyngeal cavity is primitive, not secondary (Abbott, 1953). In *S. reptans*, the ridge of the neural complex placode begins to cut off as a separate tube, except at each end, where a small connection with the inner vesicle remains. Thus, the process of neural rudiment formation in *Symplegma* is

similar to that in *Metandrocarpa*. In *M. taylori*, the extreme posterior tip of the neural complex is cut off at a fairly early stage, before any trace of the brain appears (Abbott, 1953). In *S. reptans*, on the other hand, the loss of the connection occurred at stage 9, much later than the brain formation.

The dorsal ganglion of *M. taylori* arises from the zone of junction of the neural complex and the inner vesicle and also from the ventral and ventrolateral walls of the neural complex already separated from the inner vesicle anteriorly and posteriorly (Abbott, 1953). In *S. reptans* the dorsal ganglion formed only after the neural complex had been separated completely from the inner vesicle, and therefore probably not in a zone of junction like *Metandrocarpa*'s.

The pericardium, gonad, pyloric duct, and muscle cells of *S. reptans* had their cellular origin elsewhere than in the inner vesicle of the bud. The pericardium and pyloric duct call for some discussion. Earlier works described the origin of the pericardial anlage from the floor of the inner vesicle in botryllids (Oka, 1892; Pizon, 1893; cited by Abbott, 1953; Berrill, 1941, 1947) and in *S. viride* (Berrill, 1940). Abbott (1953) stated that there is no doubt that in *M. taylori* the pericardial anlage arises from the inner vesicle, although he did not observe the very beginning of formation of the pericardial rudiment. On the other hand, recent workers do not believe that the pericardium evaginates from the inner vesicle of *Botryllus* (*e.g.*, Izzard, 1973; Nunzi *et al.*, 1979). And in *P. misakiensis* the pericardium arises as mesenchymal cell mass that has no contact with the inner vesicle (Kawamura and Nakauchi, 1984). Our study of *S. reptans* also indicated a mesenchymal origin of the pericardium.

According to Abbott (1953), the pyloric duct arises from the pyloric caecum and grows forward, penetrating to the mesenchyme surrounding the intestinal loop in *M. taylori* and *Botryllus*. Our data for *S. reptans* are inconsistent with his account. In *S. reptans* the pyloric duct and gland around the intestine are clearly derived *in situ* from the mesenchymal cell mass, although we do not deny the existence of the duct in the vicinity of the pyloric caecum. We have observed that in *B. simodensis* (in prep.), cells with high mitotic activity fuse around the intestine to form tubular structures.

In *Botryllus* the gonadal rudiment appears at the closed vesicle stage (Berrill, 1941). In this genus developing oocytes and an undifferentiated cell mass from which the testis will develop migrate from parental buds to their youngest budding offspring (Mukai and Watanabe, 1976). The germ cells are lodged in the gonadal space between the epidermis and the atrial epithelium just posterior to the budding zone. In *S. reptans* the gonadal rudiment appeared in a rather advanced bud of stages 7–8. It was lodged in the genital cavity, a concavity of the atrial epithelium, which is absent in *Botryllus*. The gonadal rudiment took a spherical, hollow shape and formed directly both sperm and ova and their accessory cells. This single origin of male and female elements contrasts with the dual origin in *Botryllus*. The difference in gonad formation between *Botryllus* and *Symplegma* may be closely related to the differing lifespans of individual zooids in these two genera.

Conclusion

In *S. reptans*, thickened cells of the bud primordium (stage 1) occupy exclusively the right side of a double-walled bud vesicle (stage 3–4). The cell thickening is, then, restricted to the right anterior atrial epithelium, budding domain of the next generation, of a stage 6 bud, suggesting that the budding activity may be transmitted through successive asexual generations. A bud vesicle of stage 3 has the long axis that coincides nearly with the anteroposterior axis appearing at stage 4. But, posteriorly the A-P axis is skewed toward the left side of the long axis, showing that the bilateral asymmetry

emerges from the very start. The body organization is constructed mainly by inward and outward foldings of the bud's inner vesicle. The neural complex placode is the first organ rudiment to be established on the inner vesicle (stage 5), which contrasts with its relatively late appearance in *Botryllus*. The pharyngeal and gut rudiments follow the neural rudiment. The pericardium, gonad, pyloric duct, and muscle cells appear first as small aggregates of mesenchymal cells. It is unknown whether the cell mass of respective organ rudiments originates from single stem cells or from heterogenous subpopulations. The gonadal rudiment of *Symplegma* zooids, unlike *Botryllus* zooids, produces both male and female elements.

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